Histamine-Induced Phosphorylation of the Regulatory Light Chain of Myosin II Disrupts the Barrier Integrity of Corneal Endothelial Cells

Sangly P. Srinivas, Minati Satpathy, Ying Guo, and Vasuki Anandan

PURPOSE. To investigate histamine-induced changes in the phosphorylation of myosin light chain (MLC) and its influence on the barrier integrity of corneal endothelial cells through altered contractility of the actin cytoskeleton.

METHODS. Experiments were performed in cultured bovine corneal endothelial cells (BCECs). Phosphorylation of MLC, which increases contractility of the actin cytoskeleton through actomyosin interaction, was assessed by urea-glycerol gel electrophoresis and Western blot analysis. Immunocytochemistry was used to locate phosphorylated MLC in relation to tight junctions. Phosphorylation of the 17-kDa PKA-potentiated inhibitory protein of type 1 protein phosphatase (CPI-17), which inhibits MLC phosphatase, was studied using Western blot analysis. The cortical actin cytoskeleton was visualized by staining with Texas-red phalloidin. Barrier integrity was determined by quantifying horseradish peroxidase (HRP; 44 kDa) flux across cells grown on porous filters.

RESULTS. RT-PCR and Western blot analysis confirmed the expression of Gqα11-coupled H1 receptors in BCECs. Exposure to histamine (100 μM; 10 minutes) led to phosphorylation of MLC (134% relative to untreated cells) and of CPI-17. Histamine also increased the flux of HRP by sevenfold and disrupted the assembly of the dense cortical actin found in resting cells. PKC activation by phorbol 12-myristate 13-acetate (PMA; 100 nM; 30 minutes) caused phosphorylation of both MLC and CPI-17. The histamine-induced MLC phosphorylation was reduced by pre-exposure to either ML-7 (50 μM), an MLCK (MLC kinase) inhibitor, or chelerythrine (10 μM), an inhibitor of PKC. Co-treatment with agents that elevate CAMP in BCECs prevented the histamine-induced MLC phosphorylation and the disruption of the actin cytoskeleton, and increased HRP flux. Phosphorylated MLC in response to histamine or PMA was found in a punctate form in close proximity to ZO-1, a marker of the tight junctional complex.

CONCLUSIONS. Histamine induces MLC phosphorylation by activating MLCK and partly inhibiting MLC phosphatase. The latter is facilitated by the phosphorylation of CPI-17. Localization of phosphorylated MLC in proximity to ZO-1 suggests increased contractility of the cortical actin at the tight junctional complex. This contractility oppose the tethering forces and lead to a breakdown of the barrier integrity. Last, elevated CAMP prevents histamine-induced loss of the barrier integrity, not only by blocking inactivation of MLC phosphatase but also by inactivating MLCK. (Invest Ophtalmol Vis Sci 2006;47: 4011–4018) DOI:10.1167/iovs.05-1127

The endothelial monolayer on the posterior surface of the cornea maintains stromal hydration, which is necessary to confer transparency to the tissue.1–3 In addition to the fluid-transport function,4 the barrier integrity of the corneal endothelium (CE) is critical for controlling stromal hydration.3,5 Recently, we demonstrated the importance of the contractility of the actin cytoskeleton in regulating the barrier integrity of the CE.6–8 An increase in the contractility of the actin cytoskeleton through phosphorylation of the regulatory myosin light chain (MLC) opposes the intercellular tethering forces at the tight junctions.9–14 Such tethering forces are necessary for interactions of transmembrane proteins associated with tight junctions which occlude the paracellular space.

MLC phosphorylation is a balance of two opposing pathways5,15: MLCK (MLC kinase)-driven phosphorylation and MLCP (MLC phosphatase)-mediated dephosphorylation. The MLCK pathway is a Ca2+-sensitive mechanism, as it activates when Ca2+-calmodulin binds to MLCK. In contrast, the MLCP-mediated pathway is independent of Ca2+. The activity of MLCP is determined by its catalytic (PP1Cδ; 38 kDa) and regulatory myosin binding (MYPT1; 110–130 kDa) subunits. Rho kinase, a downstream effector of RhoA (RhoA-Rho kinase axis), phosphorylates MYPT1 at Thr-696 and Thr-850 and thus inhibits PP1Cδ. Phosphorylation of CPI-17 (PKC-potentiated inhibitory protein of 17 kDa16,17) at Thr-38 by PKC also inhibits PP1Cδ. As a result, activation of either PKC or Rho kinase leads to MLC phosphorylation through inactivation of MLCP.15,16 Therefore, modulating the activity of MLCK and Rho kinase regulates the phosphorylation of MLC. PKA induces MLC dephosphorylation by inactivation of MLCP through its phosphorylation. However, recent studies have also suggested that PKA inhibits RhoA activation through yet unknown mechanisms.18–20

Recently, we examined cell signaling pathways affecting MLC phosphorylation to delineate pathophysiological factors that may affect the barrier integrity of the corneal endothelium. In our first study, we showed that the thrombin activates RhoA-Rho kinase axis through Gq11-coupled PAR-1 receptors in bovine corneal endothelial cells.6 The resulting increase in MLC phosphorylation led to a disruption of the cortical actin cytoskeleton with a concomitant breakdown of the barrier integrity. Pretreatment with ML-7, a selective inhibitor of MLCK, could not block the thrombin-induced MLC phosphorylation. This suggests the dominance of the Ca2+-independent pathway downstream of PAR-1 activation.6 In a sequel to this study, we demonstrated that adenosine suppresses thrombin-induced MLC phosphorylation through elevated CAMP7 presumably through inactivation of RhoA-Rho kinase axis.18–20 This study is a follow-up on these observations and has focused on the Ca2+- and PKC-dependent mechanisms involved in MLC phosphorylation during activation of H1 receptors. H1 receptors are coupled to Gq11 G-protein and are known to...
activate RhoA–Rho kinase axis. Specifically, we have investigated the histamine-induced MLC phosphorylation to explore further the influence of MLCK, PKC, and PKA on the status of MLC phosphorylation. The results show that histamine-induced MLC phosphorylation and the resultant loss of endothelial barrier integrity are suppressed by ML-7, chelerythrine, and elevated cAMP. These results emphasize a strong role for the actin cytoskeleton in the regulation of the endothelial barrier integrity and also suggest mechanisms by which agonists and antagonists of G-protein–coupled receptors (GPCRs) can affect the barrier function.

**Materials and Methods**

**Drugs and Chemicals**

Bovine calf serum was purchased from Hyclone (Logan, UT). Bovine o-thrombin and ML-7 were supplied by Calbiochem (La Jolla, CA). An enhanced chemiluminescence kit was obtained from GE Healthcare (Piscataway, NJ) and Texas red conjugated phalloidin, goat anti-rabbit Alexa 488, 4′,6-diamino-2-phenylindole (DAPI), and anti-fade agent were from Invitrogen (Eugene, OR). The polyclonal MLC antibody used for urea gelatin gel was generously provided by Patricia J. Gallagher (IUPUI, School of Medicine, Indianapolis, IN). Phosphospecific (Thr-18/Ser-19) anti-MLC antibody was obtained from Cell Signaling (Beverly, MA); anti-phospho CPI-17 (Thr-38) from Santa Cruz Biotechnology (Santa Cruz, CA); H1 receptor antibody and ZO-1 antibodies from Chemicon International (Temecula, CA); and cell culture supplies from Invitrogen-Gibco (Grand Island, NY). All other drugs/reagents were from Sigma-Aldrich (St. Louis, MO).

**Cell Culture**

Primary cultures of BCECs from fresh eyes were established in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% fetal calf serum and an antibiotic-antimycotic mixture (penicillin, 100 U/mL, streptomycin 100 μg/mL, and amphotericin-B 0.25 μg/mL).21,22 Cells were cultured at 37°C in a humidified atmosphere containing 5% CO2 and 95% air. Second- or third-passage cultures, grown to confluence on glass coverslips, porous filters (Transwell; Corning Inc., Coburn, MA), and 95% air. Second- or third-passage cultures, grown to confluence on glass coverslips, porous filters (Transwell; Corning Inc., Coburn, MA), or Petri dishes were used. Cells were starved of serum for at least 12 to 16 hours before use.

**Detection of Phosphorylation of MLC and CPI-17 by Immunoblot Analysis**

MLC phosphorylation was assayed by urea-gelatin gel electrophoresis followed by immunoblot analysis, as described previously.6,7 Protein extracts were dissolved in a urea sample buffer containing 8 M urea. The samples (25 μg/lane) were electrophoresed in polyacrylamide gels containing acrylamide, bis-acrylamide, glycerol, Tris-base, and glycine. The phosphorylated and nonphosphorylated MLC were detected by immunoblot analysis with a polyclonal anti-MLC antibody (Cell Signaling) and ZO-1 antibodies from Sigma-Aldrich (St. Louis, MO).

**Western Blot Analysis for H1 Receptors**

A purified polyclonal antibody directed against H1R (1:1000 dilution; Chemicon International, Temecula, CA), known to recognize rat H1 receptors was used in our studies. Cells grown on petri dishes were lysed with an ice-cold lysis buffer containing 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% Triton X-100, 2 mM EGTA (pH 8.0), EDTA, DTT, polyvinylidene difluoride (PVDF), Na2F, and a protease inhibitor cocktail. After a preclearing centrifugation step, the lysate was subjected to 10% SDS-PAGE, followed by immunoblot analysis. Membranes were blocked with TBST containing 5% nonfat dry milk for 1 hour at room temperature and subsequently incubated overnight at 4°C with the primary antibody diluted in TBST and dry milk. After three washes with TBST, the blots were visualized with the peroxidase-conjugated secondary antibody and enhanced chemiluminescence kit (GE Healthcare).

**Data Analysis**

One-way ANOVA was used to compare the mean results of different treatments, followed by the Bonferroni posttest analysis (Prism 4.0).
for Windows; GraphPad Software Inc., San Diego, CA). $P < 0.05$ was considered statistically significant. Data were expressed as the mean ± SE.

**Results**

**Expression of $G_{q/11}$-Coupled H1 Receptors**

Crawford et al.\textsuperscript{24,25} showed histamine-induced Ca$^{2+}$ mobilization characteristic of $G_{q/11}$ coupled H1 receptors in human and bovine CE. Accordingly, we examined the expression of H1 receptors in BCECs at protein and mRNA levels. Typical results from Western blot analysis and RT-PCR are given in Figure 1. The band in Figure 1A corresponds to the H1 receptor protein (55 kDa). The band at 758 bp in Figure 1B corresponds to the expected size for H1 receptors. After purification and sequencing of the RT-PCR product, BLAST analysis showed a primary nucleotide match to bovine H1 receptors with sequence identity of 98% over the 758-nucleotide region.

**Histamine-Induced MLC Phosphorylation**

Figure 2A is a typical gel showing the migration pattern of the di-, mono-, and nonphosphorylated forms of MLC in response to histamine. Cells were serum starved overnight (usually 12 to 16 hours) before the 10-minute exposure to histamine (100 $\mu$M). The gels were quantified by densitometry and the resultant data are summarized in the histograms of Figure 2B. Because the total phosphorylation is calculated for each lane independently, %MLC phosphorylation (denoted by %pMLC) in Figure 2B is independent of protein loading. Thus, the data shown in Figure 2B indicate significant MLC phosphorylation in response to histamine (H: 133%,$n=10$). The data also show that the histamine-sensitive phosphorylation is reduced by pretreatment with 50 $\mu$M ML-7 (ML-7: 103%,$n=7$). Chelerythrine at 10 $\mu$M also reduced histamine-induced MLC phosphorylation (Ch: 77%,$n=4$) as shown in Figure 3. These findings are consistent with histamine-induced mobilization of Ca$^{2+}$ and activation of PKC through $G_{q/11}$. Furthermore, acute exposure to phorbol 12-myristate 13-acetate (PMA), which is known to cause PKC activation, also led to MLC phosphorylation ($P$: 139%,$n=7$; Fig. 4).

**Phosphorylation of CPI-17**

CPI-17 inhibits PP1C when phosphorylated at Thr-38 by PKC.\textsuperscript{26,27} To examine the role of the PKC pathway in BCECs, we determined the phosphorylation of CPI-17 in response to

**Figure 1.** Expression of histamine receptors. (A) Western blot analysis showing the expression of H1 receptors (H1R; 55 kDa). (B) RT-PCR analysis indicating positive band at the expected size of 758 bp for H1R.

**Figure 2.** Effect of histamine on MLC phosphorylation: Cells were exposed to histamine (100 $\mu$M) for 10 minutes. Samples were separated by urea glycerol gel electrophoresis, transferred to nitrocellulose, and probed with polyclonal anti-MLC antibody for the nonphosphorylated (NP), monophosphorylated (P), and diphosphorylated (2P) forms of MLC. (A) Typical response showing MLC phosphorylation and its inhibition by ML-7 (50 $\mu$M). Cells were pre-exposed to ML-7 for 30 minutes. (B) Densitometric analysis of gels similar to that shown in (A). C, control (no treatment); H, histamine; C versus H: $P < 0.001$; C versus ML-7: $P < 0.05$; H versus H + ML-7: $P < 0.001$.

**Figure 3.** Effect of PKC inhibitor on histamine-induced MLC phosphorylation: Cells were exposed to histamine with and without the PKC inhibitor chelerythrine (10 $\mu$M). (A) Typical response showing MLC phosphorylation and its inhibition by chelerythrine. Cells were pre-exposed to chelerythrine for 10 minutes. (B) Densitometric analysis of gels similar to that shown in (A): C, control (no treatment); Ch, chelerythrine; C versus Ch: $P < 0.05$; C versus H: $P < 0.05$; H + Ch versus H: $P < 0.05$.  

$\text{Figure 3.}$ Effect of PKC inhibitor on histamine-induced MLC phosphorylation: Cells were exposed to histamine with and without the PKC inhibitor chelerythrine (10 $\mu$M). (A) Typical response showing MLC phosphorylation and its inhibition by chelerythrine. Cells were pre-exposed to chelerythrine for 10 minutes. (B) Densitometric analysis of gels similar to that shown in (A): C, control (no treatment); Ch, chelerythrine; C versus Ch: $P < 0.05$; C versus H: $P < 0.05$; H + Ch versus H: $P < 0.05$.  

$\text{Figure 1.}$ Expression of histamine receptors. (A) Western blot analysis showing the expression of H1 receptors (H1R; 55 kDa). (B) RT-PCR analysis indicating positive band at the expected size of 758 bp for H1R.
histamine and other agents known to stimulate PKC. Typical responses to histamine, thrombin, and PMA (a direct activator of PKC) are shown in Figure 5. All the tested agents induced phosphorylation of CPI-17 consistent with MLC phosphorylation, as mentioned earlier.

Effect of Elevated cAMP on MLC Phosphorylation

PKA induces MLC dephosphorylation partly through MLCK inactivation. It can also block activation of the RhoA-Rho kinase axis. To determine whether histamine-induced MLC phosphorylation is blocked by PKA, we investigated the effect of cAMP-enhancing agents on MLC phosphorylation. Cotreatment of BCECs with forskolin reduced the histamine-induced MLC phosphorylation to 86% ($\frac{H}{F}$). Similarly, adenosine, which is known to activate A2B receptors, also reduced the histamine-induced MLC phosphorylation to 83% ($\frac{H}{Ado}$). Typical gels and densitometric data for forskolin and adenosine are summarized in Figures 6 and 7, respectively.

Effect of Histamine on the Barrier Integrity and Actin Cytoskeleton

To examine changes in the barrier integrity, we measured apical to basolateral flux of HRP (44 kDa) across BCEC monolayers grown on porous filters (Transwell; Corning, Inc.). As summarized in Figure 8, histamine enhanced the level of HRP in the basolateral chamber, suggesting a breakdown of the barrier integrity. The same figure also shows that the breakdown was rescued by pre-exposure to ML-7, chelerythrine, or adenosine. The exposure to PMA also resulted in a loss of barrier integrity, which is consistent with the MLC phosphorylation noted after 30 minutes of treatment (Fig. 4).

As shown in Figure 9, resting cells show a characteristic dense assembly of actin cytoskeleton in the periphery. This

![Graph](image)

**Figure 4.** Effect of PMA on MLC phosphorylation: Cells were exposed to PMA (100 nM, 30 minutes) with and without the PKC inhibitor chelerythrine. (A) Typical response showing MLC phosphorylation and its inhibition by chelerythrine. Cells were pre-exposed to chelerythrine for 10 minutes. (B) Densitometric analysis of gels similar to that shown in (A). C, control (no treatment); Ch: chelerythrine; P, PMA; C versus Ch: $P > 0.05$; C versus PMA: $P < 0.01$.

![Graph](image)

**Figure 5.** Effect of histamine on CPI-17: Treatment with histamine (100 μM, 10 minutes), thrombin (2 units/mL; 10 minutes), and PMA (100 nM, 30 minutes) induced phosphorylation of CPI-17 (protein kinase C potentiated inhibitory protein of 17 kDa). C, control; H, histamine; P, PMA; T, thrombin.

![Graph](image)

**Figure 6.** Effect of elevated cAMP on histamine-induced MLC phosphorylation: Cells were exposed to histamine (100 μM, 10 minutes), with and without forskolin (10 μM; 10 minutes). (A) Typical response showing MLC phosphorylation and its inhibition by forskolin. (B) Densitometric analysis of gels similar to that shown in (A). C, control (no treatment); F, forskolin; C versus H: $P < 0.01$; H+F versus H: $P < 0.001$.

![Graph](image)

**Figure 7.** Effect of adenosine on histamine-induced MLC phosphorylation: Cells were exposed to histamine with and without the A2B agonist adenosine (100 μM). (A) Typical response showing MLC phosphorylation and its inhibition by adenosine. (B) Densitometric analysis of gels similar to that shown in (A). C, control (no treatment); Ado, adenosine; C versus Ado: $P < 0.001$; C versus H: $P < 0.001$; H+Ado versus H: $P < 0.001$. 

![Graph](image)
cortical assembly was disrupted in response to histamine with formation of intercellular gaps (Fig. 9; Histamine). This disruption by histamine was prevented when cells were preincubated with adenosine (100 μM; 10 minutes; Fig. 9; H + Adenosine).

**Localization of Phosphorylated MLC**

To understand how contractility of the actin cytoskeleton affects the paracellular permeability, we explored the potential mechanical linkage between the cortical actin and tight junctions. Specifically, we examined the localization of phosphorylated MLC in relation to ZO-1, which is a marker of the tight junctional complex. It is evident from the ZO-1 localization in Figure 10 that phosphorylated MLC is also located in the plane of the tight junctional complex (Histamine), in contrast to the diffuse distribution of phosphorylated MLC found under resting conditions (Control). It should be noted that the punctate localization of phosphorylated MLC along the periphery is a
common response to PMA, thrombin, and histamine (Fig. 10; PMA, Thrombin).

**DISCUSSION**

Recent studies from our laboratory have suggested a linkage between the barrier integrity of CE and its actin cytoskeleton. Specifically, we showed that agents that increase contractility of the actin cytoskeleton break down the barrier’s integrity and vice versa. This observation, which is widely reported in vascular endothelium, is consistent with the notion that an increase in the contractility of the cortical actin could oppose the intercellular tethering forces at the tight junction. The resultant imbalance of forces could disrupt the interactions of the transmembrane tight junctional proteins, which would necessarily cause a breakdown of the barrier integrity. We conducted this study to understand further the regulation of barrier integrity in the CE in terms of cell signaling pathways that affect MLC phosphorylation. Our major findings were as follows: (1) Histamine induces MLC phosphorylation, which can be suppressed by inhibitors of MLCK or PKC. MLC phosphorylation can also be inhibited by elevated camp. (2) The histamine-induced MLC phosphorylation leads to breakdown of the endothelial barrier’s integrity. These findings are not only important in helping understand the pathophysiological events that cause damage to the barrier integrity in the CE, but are also valuable for the development of pharmacologic strategies to treat the breakdown.

**Effect of MLC Phosphorylation on Actin Cytoskeleton and Barrier Integrity**

The principal effect of MLC phosphorylation is the mobilization of actomyosin interaction, which imparts increased contractility to the actin cytoskeleton which is necessary for several biological activities, including maintenance of cell shape, cell migration, cell adhesion, and cytokinesis. As shown in Figure 9, the actin cytoskeleton in CE is organized to form a cortical band. This particular pattern of the actin assembly has been referred to as the perijunctional actomyosin ring (PAMR). Most important, PAMR links to the tight junctional complex. In consistence with the histamine-induced

**Effect of PKC Inhibition on Histamine-Induced MLC Phosphorylation**

PKC is also known to inhibit MLCP (Fig. 11) However, unlike the inhibition by the RhoA–Rho kinase axis, PKC-mediated inhibition is through phosphorylation of CPI-17 rather than phosphorylation of MYPT1. It is reported that CPI-17 is phosphorylated by PKCa and PKCa isofoms and the phosphorylated form inhibits PP1C (Fig. 11). In consistence with these observations, we found CPI-17 phosphorylation in response to histamine and other PKC activating agents such as thrombin and PMA (Fig. 5). Furthermore, histamine- and PMA-induced MLC phosphorylation were reduced by the PKC inhibitor, chelerythrine (Fig. 4). These data suggest that histamine-induced MLC phosphorylation is partly through PKC-sensitive inhibition of MLCP in addition to activation of MLCK (Fig. 11).

**Effect of MLCK Inhibition on Histamine-Induced MLC Phosphorylation**

Unlike thrombin-induced MLC phosphorylation in the CE, we found the histamine-induced response to be sensitive to ML-7 (Fig. 2). We have attributed the lack of ML-7 sensitivity during thrombin response to activation of the RhoA–Rho kinase axis consistent with the activation of PAR-1 receptors coupled to Ga12/13 G-proteins. Because H1 receptors are coupled to Gaq/11 G-protein, significant activation of MLK is possible through mobilization of Ca2+ (Fig. 11). Furthermore, in the absence of significant activation of the RhoA–Rho kinase axis (compared to that observed in response to thrombin) in response to histamine, ML-7 mediated inhibition of MLCK is evident in Figure 2.
MLC phosphorylation (Fig. 3) and the resultant increase in actin contractility, we observed the disruption of the cortical actin assembly (Fig. 9) and the disappearance of the PAMR. In fact, the increased contractility has been strong enough to cause interendothelial gaps (Fig. 9; arrows in histamine) and consequently a profound increase in HRP flux (Fig 8; >sevenfold.). In consonance with these arguments, we observed phosphorylated MLC to compartmentalize in a punctate pattern to the cortical regions close to the tight junctional complex (Fig. 10). This observation highlights the importance of the equilibrium between the centripetal forces and the intercellular tethering forces at the locale of the tight junction complex. The delicate control of this mechanical equilibrium facilitates the interactions of transmembrane the tight junction proteins which occlude the paracellular space.

**Effect of Elevated cAMP on MLC Phosphorylation**

Riley et al. showed that elevated cAMP in the CE promotes deturgescence of swollen rabbit corneas and reduces the steady state thickness of fresh corneas. In a subsequent study, they demonstrated that deturgescence is promoted by elevated cAMP through an increase in the barrier integrity of the CE rather than enhanced active fluid transport. The same finding was made when the rabbit corneal endothelium was exposed to rolipram, a cAMP-dependent phosphodiesterase inhibitor. However, the mechanism underlying the increased barrier integrity in response to elevated cAMP has not been elucidated. In the previous two studies, as well as this one, we attempted to obtain a mechanistic basis for cAMP-induced enhancement in the barrier integrity. In vitro studies showed that when MLCK is phosphorylated by PKA, the MLCK complex (i.e., MLCK-Ca²⁺-calmodulin complex) loses its affinity for MLC. In addition, PKA can induce relaxation of the actin cytoskeleton by inhibiting MLCK-dependent MLC phosphorylation (Fig. 11). Given the inhibition of histamine-induced MLC phosphorylation by ML-7 (a MLCK inhibitor; see Fig. 2), we suggest that the decrease in MLC phosphorylation by exposure to forskolin (Fig. 6) or adenosine (Fig. 7) is partly due to an inhibition of MLCK through its phosphorylation by PKA.

In addition, inhibition of MLC phosphorylation is also noticeable when RhoA activation is suppressed as shown in our previous studies. When MYPT1 is phosphorylated by Rho kinase (a downstream effector of RhoA) at Thr-696 and Thr-850, the phosphatase activity of MLCP contained in PP1C is inhibited. This phosphorylation abrogates RhoA signaling, leading to MLC dephosphorylation as seen with agents that increase cAMP levels (Figs. 6, 7; also, Fig. 11).

In summary, this study shows that histamine induces a loss of barrier integrity in CE by increasing MLC phosphorylation through Ca²⁺ and PKC-mediated pathways (Fig. 11). This effect is promptly overcome by elevated cAMP (Fig. 11). The findings indicate a causal link between MLC phosphorylation and breakdown of the barrier integrity in the CE.

**References**